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I, LEANNE MYNOTT, MANAGER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2002950658 for a patent by THE CORPORATION OF THE TRUSTEES OF THE SISTERS OF MERCY IN QUEENSLAND as filed on 08 August 2002.

WITNESS my hand this
Nineteenth day of August 2003

LEANNE MYNOTT
MANAGER EXAMINATION SUPPORT
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AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"A method of immunomodulation"

The invention is described in the following statement:

[redacted]

A METHOD OF IMMUNOMODULATION

FIELD OF THE INVENTION

The present invention relates generally to a method for modulating the immuno-activity of an antigen-presenting cell and agents useful therefor. More particularly, the present invention relates to a method for preventing or down-regulating one or more functional activities of a dendritic cell. The present invention further provides antibodies and, in particular, monoclonal antibodies, which interact specifically with epitopes present on the surface of dendritic cells, resulting in depletion, down-regulation or destruction of targeted dendritic cell *in vivo* or *in vitro*. The instant invention further provides a method for modulating an immune response in a subject and, in particular, for down-regulating the immuno-activity of an allogeneic immuno-competent graft and/or the immune response of a recipient of a solid organ transplant. The ability to modulate dendritic cell immuno-activity may be useful, *inter alia*, in a range of immuno-therapeutic and immuno-prophylactic treatments that benefit from immune suppression.

BACKGROUND OF THE INVENTION

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

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Dendritic cells (DC) are potent cellular activators of primary immune responses (Hart, *Blood 90:* 3245-3287, 1997). Immature myeloid DC in non-lymphoid organs react to, endocytose and process antigens and migrate *via* blood and lymph to T cell areas of lymphoid organs. Here, the mature cells present foreign peptide complexed to MHC Class

II to T cells and deliver unique signals for T-cell activation (immuno-stimulation). They also stimulate B lymphocytes and NK cells. DC undergo differentiation /activation during this process, lose their antigen-capturing capacity and become mature, immuno-stimulatory DC that trigger naïve T-cells recirculating through the lymphoid organs. The lymphoid DC subset may have a different migration pathway and although capable of stimulating allogeneic and autologous T-lymphocytes they have been suggested to have a regulatory function (Grouard *et al., J. Exp. Med.* 185: 1101-1111, 1997). As part of the differentiation/activation process, DCs up-regulate certain relatively selectively-expressed cell surface molecules such as the CMRF-44 and CD83 antigens. DC in the thymus and DCs that do not have an activated co-stimulating phenotype probably contribute to central and peripheral tolerance.

Allogeneic transplantation involves the transfer of material from a host to a recipient. In this process, many foreign antigens are introduced into a host and an immune response results when these foreign antigens are detected by the host's immune system. Initially, an immune response involves interactions between the antigen and antigen-presenting cells (APC) such as dendritic cells. *Interstitial* donor DC in heart and kidney contribute to (direct) recipient T lymphocyte sensitization to all antigens but recipient DC, after migrating into the donor tissue, can also stimulate (indirect) alloantigen sensitization of recipient T-lymphocytes. Depletion of heart and kidney and pancreatic islet DC appears to prolong allograft survival. Interestingly, during liver transplantation, donor leucocytes, which may include non-activated dendritic cells, appear to generate allogeneic tolerance. DC are also predicted to contribute to both acute and chronic Graft *Versus* Host Disease (GVHD), the major life threatening complication of allogeneic bone marrow transplantation (BMT). Blood DC counts change during acute GVHD and recent data have suggested that the DC subset constitution of the allogeneic stem cell preparation might relate to GVHD outcome. Recent evidence from a mouse model suggests that host APC contribute to the acute GVHD. DC may in certain circumstance prevent acute GVHD.

Monoclonal antibodies (mAb) which act at the level of the responder T lymphocyte have been investigated as therapeutic immunosuppression agents in allogeneic transplantation.

The CD3 reagent OKT3 *(Orthoclone, Cilag)* is used routinely to treat acute renal allograft rejection. *Campath* 1 (CD52) and its variants have been used in solid organ transplant and BMT. More recent attempts to suppress acute GVHD have involved the antibody ABX-CBL (CD147) (Deeg *et al., Blood 98:* 2052-2058, 2001) and anti-IL-2R mAb Daclizumab (Cahn *et al., Transplantation 60:* 939-942, 1995). Attempts to interfere with the interaction of the responder T-lymphocyte and an APC have focused on antibodies directed against the co-stimulator molecules CD40, CD80 and CD86 or their ligands. Animal studies suggest that blockade of co-stimulator molecules on DC and other APC induces T cell anergy and prolongation of solid organ grafts (Koenen and Joosten, *Blood 95:* 3153-3161; 2000, Kirk *et al., Nat. Med. 5:* 686-693, 1999; Kirk *et al., Proc Natl Acad Sci USA 94:* 8789-8794, 1997). The use of CD80, CD86 and CD28 blocking agents prevents acute GVHD in mice (Blazar *et al., Blood 85:* 2607-2618, 1995) and *in vitro* blockage of allogeneic responses in allogeneic stem cell preparations has been used in clinical BMT with initial encouraging results (Gribben *et al., Blood 87:* 4887-4893, 1996). The use of a reagent that was more selective at targeting differentiated/activated DC might be advantageous.

In humans, at least two populations of DC, the immature myeloid DC and the plasmacytoid DCs, have been identified based on differential expression of CD11c (O'Doherty *et al., J Exp Med 178*: 1067, 1993; O'Doherty *et al., Immunol 82:* 487, 1994) More recent studies have shown that $CD11c^-$ DC have a different phenotype and express higher amounts of CD123, and have a morphology and function distinct from $CD11c^+$ DC (Grouard *et al., J Exp Med. 185:* 1101-1111, 1997). These two subsets are denoted as myeloid lineage $CD11c^+$ DC and plasmacytoid $CD123^+$ DC. It is thought unlikely that there is a direct developmental relationship between them (Robinson *et al., Eur J Immunol 29:* 2769, 1999).

Theoretically, mAb directed at DC administered to the recipient of a solid organ graft would deplete donor DC (i.e. direct) as well as recipient DC (indirect) as they enter the circulation and initiate antigen presentation pathways. Other donor leucocytes may have immunomodulatory capacity. DC depletion therapy might then be ceased after a short

period, allowing tolerance to emerge. Depleting recipient DC may be more efficacious than disrupting co-stimulator pathways. Investigation of this concept has been delayed, however, by the absence of suitable DC reagents. CMRF-44 mAb is an antibody specific for DC and is used for the identification and isolation of human blood DC (Fearnley *et al.*, *Blood 89:* 3708-3716, 1997). The latter authors have shown that the epitope for CMRF-44 mAb (i.e. CMRF-44 Ag) is expressed early in the differentiation of DC from circulating precursor cells.

Given the importance of dendritic cells in the overall immuno-potential of an individual, there is a need to identify agents, which can facilitate modulation of DC activity.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The present invention is predicated in part on the determination that a cell-surface activation molecule may act as a target for agents, the binding of which, results in disablement and/or eventual destruction of the cell. In particular, it has been shown that CMRF-44 mAb is capable of initiating lysis of antigen presenting cells such as DC. More particularly, CMRF-44 is capable of acting as an immuno-suppressive agent, by down-regulating DC function. Thus, the present invention provides reagents useful for the down-regulation of activated DC, and a method for the suppression of an immune response useful *inter alia* for the reduction or prevention of allogeneic graft rejections, graft *versus* host disease, and the amelioration of certain auto-immune inflammatory interactions, such as rheumatoid arthritis.

The present invention, therefore, contemplates a method for modulating the immuno-activity of an antigen-presenting cell (APC) by contacting the APC with an effective amount of an agent which couples, binds or otherwise associates with a cell-surface activation molecule and in turn prevents, inhibits or otherwise down-regulates one or more functional activities of the APC.

Generally, the APC is a DC.

Preferably, the DC is a myeloid DC and, in a particularly preferred embodiment, belongs to the $CD11c^+$ DC sub-population.

In a preferred embodiment, the agent comprises a monoclonal antibody such as, for example, CMRF-44, or a derivative, fragment, homolog, analog or chemical equivalent or

mimetic thereof and the cell-surface activation molecule is a molecule or a derivative, fragment, homolog, analog or chemical equivalent or mimetic thereof, expressed on the surface of a DC and which interacts with CMRF-44 antibody.

The present invention is further directed to a method for modulating an immune response in a subject by administering to the subject an effective amount of an agent which couples, binds or otherwise associates with an antigen presenting cell's surface activation molecule (e.g. a DC surface molecule which interacts with CMRF-44) which in turn prevents, inhibits or otherwise down-regulates one or more functional activities of the APC.

The agent of the present invention may also be used to down-regulate the immuno-activity of an immuno-competent graft such as a bone marrow graft.

Another aspect of the present invention contemplates a method for the prophylactic and/or therapeutic treatment of a condition characterized by the aberrant, unwanted or otherwise inappropriate immuno-activity of an immuno-competent graft by contacting the graft with an effective amount of the agent or a derivative, homolog, analog, chemical equivalent or mimetic thereof which prevents, inhibits or otherwise down-regulates the inappropriate immuno-activity of the graft.

The present invention further extends to pharmaceutical compositions and formulations comprising the agent for use in conjunction with the instant methods, and to the use of such agents in the manufacture of a pharmaceutical composition or formulation.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 are graphical representations showing examples of CMRF-44 expression in cultured human leukocytes. **(A)** PMBC (activated DC are defined as PE^- $FITC^+$ cells, in lower right quadrant), **(B)** purified Lin^- PBMC cultured overnight with GM-CSF and IL-4, **(C)** $CD11c^+$ Lin^- PBMC cultured as in B, and **(D)** $CD123^{hi}$ Lin^- PBMC cultured as in B. In A, the quadrant positions were determined by negative control staining. In B-D, the left hand line represents IgM negative control staining.

Figure 2 are graphical representations showing CMRF-44-specific complement-mediated DC lysis occurs in cultured human PBMC. The combination of CMRF-44 and autologous human serum (AS) deplete $CD83^+$ DC. Treatments = **(A)** AS only, **(B)** CMRF-44 mAb only, **(C)** CMRF-44 + AS, **(D)** negative control IgM + AS. Lower right quadrants show percentage of DC in treated cultured PBMC.

Figure 3 are graphical representations showing Lin^- DC survival is improved with GM-GSF and IL-3 present during overnight culture. **(A)** Cell death analyzed by PI/Annexin-V labeling after overnight culture with or without the addition of GM-CSF/IL-3. **(B)** Example of Lin^- DC, in live forward/side scatter gate showing improved yield of $CMRF\text{-}44^+$ cells after culture with GM-CSF + IL-3 (left-hand curves + IgM negative control).

Figure 4 are graphical representations showing CMRF-44-specific complement-mediated lysis of DC within a cultured purified human DC (Lin^- cell) preparation. The effect on the percentage of $CD11c^+$ $HLA\text{-}DR^+$ cells (dot plots, upper right quadrants) and on the percentage of dead $7\text{-}AAD^+$ cells (histograms) after treatment with **(A)** medium alone, **(B)** 1:2 v/v AS alone, and **(C)** 20 ug/ml CMRF-44 and AS combined is shown.

Figure 5 are graphical representations showing examples of CMRF-44 specific complement mediated lysis of cultured $CD11c^+$ and $CD123^{hi}$ DC sort purified from a Lin^- preparation. **(A, B)** $HLA\text{-}DR^+$ $CD11c^+$ DC treated with autologous human serum (AS) and either (A) negative control IgM, or (B) CMRF-44 mAb. **(C, D)** $HLA\text{-}DR^+$ $CD123^{hi}$ DC

More particularly, the present invention contemplates a method for the prophylactic and/or therapeutic treatment of a condition characterized by the aberrant, unwanted or otherwise inappropriate immuno-activity of an immuno-competent graft, in a subject, said method comprising contacting said graft with an effective amount of an agent or a derivative, homolog, analog, chemical equivalent or mimetic thereof, which agent couples, binds or otherwise associates with an APC's surface activation molecule derived from said graft, for a time and under conditions sufficient to prevent, inhibit or otherwise down-regulate the said inappropriate immuno-activity of said graft.

Preferably, the said subject is a human. Preferably, the said condition is graft *versus* host disease.

Still more preferably said graft is an allogeneic bone marrow graft, spleen cell graft or a stem cell graft.

Reference herein to "therapeutic" and "prophylactic" treatment is to be considered in its broadest context. The term "therapeutic" does not necessarily imply that a subject is treated until total recovery. Similarly, "prophylactic" does not necessarily mean that the subject will not eventually contract a disease condition. Accordingly, therapeutic and prophylactic treatment includes amelioration of the symptoms of a particular condition or preventing or otherwise reducing the risk of developing a particular condition. The term "prophylactic" may be considered as reducing the severity or the onset of a particular condition. "Therapeutic" may also reduce the severity of an existing condition.

The methods of the present invention may have further use in the prophylactic and/or therapeutic treatment of a range of other conditions characterized by an unwanted or undesirable immune response. Such conditions include, *inter alia*, those wherein the response is inappropriate as well as those wherein the response may be regarded as being physiologically normal but is nevertheless undesirable. Examples include auto-immune conditions, chronic inflammatory conditions, asthma and hypersensitivity, allergies to innocuous agents and transplant rejection.

More particularly, conditions which are proposed to be treatable using the methods of the present invention encompass auto-immune and inflammatory disorders such as, for example, rheumatoid arthritis, lupus erythematosus, systemic lupus erythematosus, Hashimotos thyroiditis, multiple sclerosis, myasthenia gravis, type 1 diabetes, anto-immune anaemia, thrombocytopenia, inflammatory bowel disease and Crohn's disease.

In any condition, where undesirable responses are triggered by the presentation of antigen, the methods of the present invention may find useful application.

Accordingly, another aspect of the present invention contemplates a method for the prophylactic and/or therapeutic treatment of a condition characterized by an aberrant, unwanted or otherwise inappropriate immune response in a subject, said method comprising administering to said subject an effective amount of an agent, which agent couples, binds or otherwise associates with an APC's surface activation molecule, for a time and under conditions sufficient to prevent, inhibit or otherewise down-regulate the immuno-activity of said APC.

The present invention further extends to pharmaceutical compositions and formulations comprising the said agents for use in conjunction with the instant methods. Such pharmaceutical compositions and formulations may be administered to a human or animal subject in any one of a number of conventional dosage forms and by any one of a number of convenient means. The agent of the pharmaceutical composition is contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the agent chosen. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 mg to about 1 mg of agent may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation.

The agent may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intraperitoneal, intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). The agent may be administered in the form of pharmaceutically acceptable non-toxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

Routes of administration include, but are not limited to, respiratorally, intratracheally, nasopharyngeally, intravenously, intraperitoneally, subcutaneously, intracranially, intradermally, intramuscularly, intraoccularly, intrathecally, intracereberally, intranasally, infusion, orally, rectally, *via* IV drip patch and implant.

In accordance with these methods, the agent defined in accordance with the present invention may be co-administered with one or more other compounds or molecules. By "co-administered" is meant simultaneous administration in the same formulation or in two different formulations *via* the same or different routes or sequential administration by the same or different routes. For example, the subject agent may be administered together with an agonistic agent in order to enhance its effects. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two types of molecules. These molecules may be administered in any order.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of

manufacture and storage and must be preserved against the contaminating action of micro-organisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The prevention of the action of micro-organisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied

and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule encoding a modulatory agent. The vector may, for example, be a viral vector.

The present invention further contemplates a combination of therapies, such as the administration to a subject of the agent of the present invention in a pharmaceutical composition or formulation together with a low dose of immuno-suppressive drugs.

Yet another aspect of the present invention is directed to the use of an agent of the present invention in the manufacture of a pharmaceutical composition or formulation for use in the method of the invention.

The present invention is further described by the following non-limiting Examples.

[redacted]

EXAMPLE 1

Material and methods

CMRF-44 Purification

CMRF-44 (IgM) was purified from conditioned tissue culture supernatant (10% w/v FCS in RPMI 1640) by dilution in an equal volume of 0.15 mol/l Na_2HPOI_4, pH 7.2 and passage through a 2ml column of Protein-L immobilized on agarose beads (Pierce #20510). The column was washed with the above buffer until eluent A_{280nm} <0.010. Bound material was eluted with 4ml 0.1 mol/l glycine at pH 2.5 and immediately neutralized with 0.4ml 1 mol/l Tris at pH 9. The protein content was estimated by A_{280nm} measurement, it contained CMRF44 immunoreactivity, and SDS-PAGE analysis under reducing conditions revealed only two bands with MW consistent with IgM H- and L- chains.

Cell Preparations: PBMC

PMBC were purified over Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) from buffy coats from volunteer donors by standard methods.

Purified Lineage Negative Blood DCs

Lineage negative cells were prepared from fresh PBMC. MACS columns (Miltenyi Biotec, Becton Dickinson, Australia) and magnetic beads (Biomag, goat anti-mouse IgG Fc, Polysciences Inc., Warrington, P.A., USA) were prepared according to the manufacturer's protocols. Briefly, a 3-way stop-cock was attached to a large CS (6.3ml) column, a 10 ml syringe filled with BSA/EDTA/PBS horizontally fitted to the stopcock, a 23 g needle inserted vertically and attached to the MACS (Vario) magnet. The end of the needle cover (attached to the needle) was clipped. The syringe was used to expel air from the needle and the column was washed by adding 35 ml of BSA/EDTA/PBS *via* the top of column. To prepare beads for addition to cells, beads were washed twice with cold 0.5% w/v BSA/2 mM EDTA/PBS (MACS buffer).

[redacted]

PBMC were stained with a prepared cocktail of monoclonal antibodies to enable removal of lineage positive cells. The lineage cocktail contained an optimized mix as follows: 25% v/v OKT3 (T cells, CD3); 15% v/v OKM1 (Mo, CD11b; 15% v/v CMRF31 (Mo, CD14); 10% v/v HUNK-2 (NK, CD16); 20% v/v FMC63 (B, CD19) All were IgG. Lin^+ cell depleting mAb mix 0.1 ml was added per 10^6 cells. The cells were mixed with the cocktail and incubated on ice with occasional shaking for 20 min. The preparation was washed twice with MACS buffer and were resuspended in washed magnetic beads (1 ml beads per 50 x 10^6 cells). The cells were incubated on ice for 15 min with gentle mixing. The suspension was cleared initially on a MPC-1 magnet (Dynal, Carlton South, Victoria, Australia) and then the supernatant was passed through a BS depletion column (Miltenyi). The eluate was centrifuged for 5 min at 4°C, 500 g and resuspended in PBS. The eluted cells were lysed with Vitalize (BioErgonomics, St Paul, MN) to remove residual erythrocytes. To check for contaminating antibody-labelled cells, the preparations were stained with FITC-conjugated sheep anti-mouse immunoglobulin (FITC-SAM) (1:50, v/v) for 10 min. Lin^- cells were identified and collected on a FACS-Vantage cell sorter, FITC positive cells being gated out. To obtain DC sub-sets, CD11c-APC and CD123-PE were added with the FITC-SAM and separated populations of $CD11c^+$ and $CD123^{hi}$ cells were sorted.

Complement Sources

Low-Tox-M Rabbit complement was obtained from Cedarlane Laboratories (Hornby, Ontario) Fresh serum (up to 24 hr), prepared by centrifugation of clotted blood, was used as autologous human complement.

PBMC Cytotoxicity Assay

PBMC (10 ml at 10^7 cells/ml) were cultured in a 90 cm petri dish (Sarstedt, Ingle Farm, South Australia) at 37°C overnight in 5% v/v CO_2 to induce expression of CMRF-44 and CD83. After Ficoll separation to remove dead cells the cells were washed and resuspended

in cytotoxicity medium (RPMI1640, 0.3% w/v BSA, 25 mM Hepes). Aliquots of the cells were stained with either CMRF-44 or negative control IgM followed by FITC-SAM, CD14-PE and CD19-PE to check for upregulation of CMRF-44 antigen on DC. As some activated B-cells and monocytes, but not T- and NK cells, also express CMRF-44 antigen, DC were defined here as PE-negative, FITC-positive events. 1.2 x 10^6 PBMC in 0.3 ml was added to each 5 ml polypropylene culture tube. Purified CMRF-44 (or control, TEPC-2 purified myeloma IgM, Sigma) was added at 20 ug/ml and the tubes were placed on ice for 20 min. Rabbit complement (50 µl) or 300 µl of either autologous human serum (AS) or heat activated autologous human serum (HIAS) was added and the tubes were cultured for 1 hr at 37°C in a 5% v/v CO_2 incubator, followed by further washing. To monitor DC depletion, aliquots were stained with CD14/19-PE and with FITC conjugates of either the independent DC marker CD83 or control antibody. DC were defined as $FITC^+$, PE^- flow cytometry events in the live forward scatter gate, and these were expressed as % of all cells in the live gate.

Purified DC (Lineage Negative) Cytotoxicity Assay

Purified DC (Lin^- cells) were cultured overnight with or without GM-CSF (200 U/ml, Schering-Plough, Sydney, NSW) and IL-3 (10 ng/ml, Invitrogen, Mulgrave, Victoria, Australia) in 0.5-1 ml of cytotoxicity medium in round bottom polypropylene culture tubes (5 ml; Falcon, BD Biosciences, North Ryde, NSW). An aliquot taken before and after overnight culture was monitored for cell death by flow cytometry (Annexin-PE and PI). To assess the percentage of $CMRF\text{-}44^+$ cells a portion of the cultured DC preparation was stained with biotinylated CMRF-44 or biotinylated IgM negative control followed by streptavidin-PE.Cy5 and either CD11c-FITC and HLA-DR-PE or CD123-PE and HLA-DR-FITC. To effect depletion, approximately 5 x 10^4 cells in each tube were stained with or without CMRF-44 (20 µg/ml, as for PBMC. Initially cells were resuspended in 500 µl of cytotoxicity medium, 25 µl of rabbit complement was added and the cells were cultured at 37°C as above for PBMC. Autologous human serum was used thereafter.

For DC subset analysis, Lin^- cells treated with CMRF-44 mAb and complement were stained with either CD11c-FITC and HLA-DR-PE (for Lin^- cells and CD11c purified cells) or with CD123-PE and HLA-DR-FITC (for CD123 purified cells). PI and Annexin-V were used to assess apoptosis in enriched DC preparations, otherwise 7-AAD was used to exclude dead cells.

Flow Cytometry DC Enumeration

TRUCOUNT tubes (BD Biosciences) were used to quantitate mAb and complement-mediated cell depletion. Purified Lin^- DCs (or $CD11c^+$, or $CD123^{hi}$ subsets) were cultured overnight with cytokines (GM-CSF and IL-3) in polypropylene tubes. Cells were washed twice in cytotoxicity medium. An aliquot of 100 µl, containing 20,000 cells was added to polypropylene tubes, 200 µl cytotoxicity medium was then added, then either 20 µl of medium or CMRF-44 or control IgM. Cells were incubated on ice for 30 min., centrifuged and 120 µl of supernatant removed. Serum or heat inactivated serum (200 µl) was added and tubes incubated at 37°C for 1 hr. Cells were centrifuged and 300 µl supernatant removed. To the 100 µl remaining, antibodies were added and tubes incubated for 20 min on ice. PBS (220 µl) was then added. After this, 300 µl of cells was transferred to TruCOUNT tubes and vortexed. Cells were left for 10 min and revortexed before counting. Data were expressed as cells per 10,000 beads.

Functional Assays

For tetanus toxoid (TT) and keyhole limpet haemocyanin (KLH) antigen presentation assays, PBMC from freshly donated blood were cultured overnight and treated as described above for the PBMC cytotoxicity assay. The washed cells were resuspended in 5% AS serum in RPMI1640 containing manufacturer's recommended quantities of HEPES, pyruvate, non-essential amino acids, penicillin and streptomycin (Invitrogen), and introduced into wells, at 1-3 x 10^5/well as required of a 96-well round bottom culture plate (Falcon) containing TT or KLH in the same medium (final volume = 200 µl/well). Plates were cultured for 6 days at 37°C in 5% v/v CO_2, then 1 µCi of ^{3}H-thymidine (Amersham,

[redacted]

Sydney, NSW) was added per well, and culture continued for a further 1 hours before harvesting (TomTec Mach III, Hamden, CT) and ^{3}H-thymidine incorporation measurement by liquid scintillation spectroscopy (Wallac, Finland). T-cell proliferation is presented as counts per minute (CPM).

For the allogeneic mixed lymphocyte reaction (MLR), PBMC treated as above with CMRF-44 and AS or HIAS were irradiated (3000 cGy) and added to wells in a round bottom 96-well plate containing 10^{5} allogeneic CD4+ CD45RA+ T-cell responders. The latter were prepared from buffy coat derived PBMC by rosette purification with neuraminidase-treated sheep red cells (and AB serum), followed by negative selection by FACS after staining with PE-conjugated mAbs for CD8, CD14, CD16, CD19, CD34, CD45RO, CD56, and HLA-DR. The purified cells were >85% CD4+ CD45RA+. The plates were cultured for 4 days, ^{3}H-thymidine labeled, and harvested 16 hours later, and analysed as above.

EXAMPLE 2

Expression of CMRF-44 on PBMC, lineage negative cells and purified DC subsets

Repeated studies confirmed the presence of a small CMRF-44^{+} DC population in cultured PBMC (Fearnley *et al., Blood 89:* 3708-3716, 1997). As purified lineage negative blood DC populations are now routinely divided into CD11c and CD123 subsets, the expression of CMRF-44 on PBMC was analyzed, lineage negative and the CD11c and CD123 subsets. (Figure 1) The CMRF-44 antigen is expressed on approximately 0.5-2.0% PBMC and on a high proportion of purified lineage negative DCs after culture. It was induced on the majority of CD11c^{+} DC and on a significant population of activated CD123^{hi} DC. These CMRF-44^{+} DC co-express the different DC activation antigen CD83 [Fearnley *et al.*, 1999, *supra*].

EXAMPLE 3

CMRF-44 and complement kills CD83$^+$ cells in PMBC

The cytotoxic effects of CMRF-44 and rabbit allogeneic and autologous complement were tested on PBMC DC populations, using a CD83 mAb to monitor the activated DC population.

Initial experiments with CMRF-44 and rabbit complement established that CMRF-44 mediated blood DC cytotoxicity. The effect titred with the antibody and occurred whether or not the cells were washed after incubation with antibody. Low concentrations (5% v/v) of rabbit complement were effective. However, despite being selected for its lack of spontaneous cytotoxicity of lymphoid cells, rabbit complement intermittently reduced the number of CD83$^+$ cells, suggesting a background cytotoxic effect on blood DC. The CMRF-44 mAb and pooled AB serum as a complement source likewise mediated lysis of CD83$^+$ cells but, again, donor variable background cytotoxicity was a problem.

Autologous human serum (AS) was tested as a complement source (Figure 2). This reduced background cytotoxicity to a consistently low level. No lysis occurred if the AS was heat inactivated (HIAS), nor did it occur if CMRF-44 was replaced by IgM negative control (Figure 2D). Seven consecutive preparations were then analyzed: the mean percentage of CD14$^-$/19$^-$CD83$^+$ cells in cultured PBMC treated with CMRF-44 and HIAS was 0.50% (SD = 0.16.). CMRF-44 plus AS treatment reduced this to a mean of 0.06% (SD = 0.08) ($p<0.0005$, Student's paired *t*-test). This and the data in Figure 2 indicate that the cytotoxicity is highly specific.

EXAMPLE 4

Optimization of cytotoxicity assays using purified DC

Blood DCs were purified from PBMC using negative immunoselection. Initial studies showed that a high proportion of DCs in these preparations underwent spontaneous cell death when cultured overnight, which contributed to a significant cytotoxicity background

as measured by PI and Annexin-V staining. Other data indicated that the addition of cytokines would reduce background cytotoxicity of the $CD123^{hi}$ DC subset in particular and, therefore, the Lin^- DCs were cultured in GM-CSF and IL-3 overnight. This reduced background apoptosis and cell death (Figure 3A) and increased the proportion of CMRF-44^+ cells available for analysis (Figures 3B). Therefore, subsequent Lin^- DC preparations were routinely cultured overnight with GM-CSF and IL-3.

The optimal CMRF-44 mAb concentration for maximum cytotoxicity (measured both as a decrease in cells that were $CD11c^+$ and $HLA\text{-}DR^+$, and as an increase in total 7-AAD positive cells) was found to be greater than or equal to 10 μg/ml. The optimal AS concentration was found to be 1:2 v/v. These conditions were used in subsequent experiments.

To investigate the subsets of Lin^- cells, which were susceptible to CMRF-44 mediated complement lysis, the Lin^- cells were stained with 7AAD, $CD11c^-$ FITC and HLA-DR-PE. The results (Figure 4) showed that the cells of $CD11c^+$ population were profoundly reduced, accompanied by an increase in AAD positive cells. Optimization experiments, repeating the CMRF-44 titration and complement concentrations, confirmed these results.

The effect of CMRF-44 and complement on the CD123 subset within Lin^- cells was then examined. The results depended on the induction of the CMRF-44 antigen on this subset. Thus, in some cases the $CD123^+$ ($CD11c^-$) population was only partially affected (20%); in other cases a greater proportion (up to 90%) of $CD123^+$ cells was killed (Table 2).

TABLE 2 Percentage of CD11c$^+$ DR$^+$ and CD123$^+$ in lineage negative cells before and after treatment with CMRF-44.

	Percentage of positive cells present					
Treatment of Lin$^-$ cells	**CD11c**	**7AAD**	**% killed***	**CD123**	**7AAD**	**% killed***
CMRF-44 + autologous serum*	1	32	92	3	36	90
IgM + autologous serum*	16	3		30	6	
CMRF-44 + autologous HI serum	16	6		35	11	
Medium only	22	7		28	12	
1 in 1 autologous serum	17	3		36	6	
1 in 2 autologous HI serum	16	6		35	11	

* For % killed, compared IgM and CMRF-44 with autologous serum.

Cells were stained with either CD11c-FITC, HLA-DR-PE and 7 AAD or CD123-PE, HLA-DR-FITC and 7 AAD.

EXAMPLE 5

Absolute counts to document CMRF-44 cytotoxic effects

TruCOUNT bead methodology was introduced to monitor DC depletion accurately (see Example 1). This confirmed that both CD11c$^+$ and CD11c$^-$ (containing CD123^{hi}) populations were susceptible to CMRF-44 and AS treatment. An example is shown in Table 3.

TABLE 3 TruCOUNT analysis of CMRF-44 mediated cytotoxicity on lineage negative sorted cells after overnight culture and treated with CMRF-44 and autologous serum.

	No. of cells (events) per 10,000 beads in each quadrant				
Treatment of Lin⁻ cells	**UL (DR^+11c^-)**	**UR (DR^+11c^+)**	**LL (DR^-11c^-)**	**LR (DR^-11c^+)**	**Total**
CMRF-44 + AS*	280	111	272	56	819
IgM + AS*	347	573	373	63	1356
CMRF-44 + HI AS	370	452	254	74	1150
Medium only	472	474	195	11	1152
AS only	182	525	191	87	985
HI AS only	419	402	260	14	1095
CMRF-44 only	220	633	223	89	1165
IgM (PEPC83) only	512	457	171	6	1146

* Comparing CMRF-44 +AS with IgM +AS then 40% cells died. Most of the cells dying were $CD11c^+DR^+$.

Cultured lineage negative cells were treated with CMRF-44 + AS, IgM +AS, CMRF-44 + HI AS, medium only, autologous serum 1 in 2 (AS) only, heat inactivated (HI) AS only, CMRF-44 only, IgM only. 7AAD+ cells gated out. Cells stained with CD11cFITC, HLADR-PE and 7AAD. Cell count 46% cells stained $CD11c^+CMRF\text{-}44^+$.

The two DC subsets were sort purified, cultured separately overnight with GM-CSF and IL-3 and then treated with CMRF-44 and AS. Purified $CD11c^+$ DC were predominantly $CMRF\text{-}44^+$ after culture and the majority (<90%) of these cells were depleted by treatment with CMRF-44 and AS. Purified $CD123^{hi}$ DC were variably $CMRF\text{-}44^+$ after culture, and, after treatment, this generally resulted in a lower percentage lysis compared to $CD11c^+$ DC (n = 3 experiments, Table 4, e.g, Figure 5), but this percentage reflected near complete lysis of the $CRMF\text{-}44^+$ $CD123^{hi}$ DCs.

TABLE 4 Analysis of CMRF-44 and complement treated cultured CD11c^{+} and CD123^{hi} DC

		No. of cells per 10,000 TruCOUNT beads *Treatment*			
Experiment No.		**IgM + AS**	**CMRF-44 + AS**	**% CMRF-44^{+}**	**% depletion**
1	CD11c^{+}	941	48	97%	95%
	CD123^{hi}	452	390	60%	14%
2	CD11c^{+}	6188	427	80%	93%
	CD123^{hi}	1870	1172	40%	37%
3	CD11c^{+}	4698	171	96%	96%
	CD123^{hi}	2129	471	72%	78%

Sort purified CD11c^{+} or CD123^{hi} DC were cultured overnight with GM-CSF + IL-3 and treated with 20 ug/ml of either negative control IgM or CMRF-44 followed by autologous serum 1:2 v/v (AS) as described in Example 1. Cells were then stained with 7-AAD and either CD11c-FITC and HLA-DR-PE or CD 123-PE and HLA-DR-FITC. The 5th column shows the % of CMRF-44+ cells (stained separately) prior to AS treatment. The 6th column = 100% [1-column4 / column 3].

EXAMPLE 6

Functional effect of CMRF-44 and complement DC lysis on PBMC

Previous experiments have shown that CMRF-44^{+} DC stimulate a recall tetanus toxoid (TT) proliferative T cell response and are essential to generate a primary (KLH) response. PBMC treated with CMRF-44 and AS were, therefore, tested for their ability to present TT and KLH. A substantial and statistically significant reduction in the ability of treated PBMC, relative to heat inactivated AS controls, to stimulate a primary proliferative response to KLH was found ($p<0.05$, Figure 6). Reduced secondary responses to TT were also found, but were not as consistent or as marked (Figure 7). Background counts were frequently significantly reduced after CMRF-44 and AS treatment, confirming the central role of CMRF-44^{+} cells in the autologous mixed lymphocyte reaction.

EXAMPLE 7

Stimulation of $CD4^+$ T-lymphocyte reaction

Irradiated overnight cultured PBMC depleted of DC using CMRF-44 and AS were then tested for their ability to stimulate allogeneic $CD4^+$ $CD45RA^+$ T-cells. Statistically significant reductions in T-cell proliferation were observed, compared to heat inactivated autologous serum controls. The inhibitory effect was most substantial at low stimulator: responder ratios (Figure 8).

The CMRF-44 mAb has, in continuation with autologous complement, specific cytotoxity activity against DC which undergo differentiation/activation in cultured blood PBMC, resulting in lysis of >88% of the CD11c DC subset associated with strong T_h1 responses. The $CD123^{hi}$ DC subset, associated with T_h2 type responses but none-the-less capable of initiating a significant allogeneic response when activated, is also susceptible. These experiments establish the possibility of manipulating DC to prevent detrimental and to promote beneficial immune responses in allogeneic BMT and other forms of organ transplantation.

Those skilled in the art will appreciate that the present invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the present invention includes all such variations and modifications. The present invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

DATED this eighth day of August 2002.

The Corporation of the Trustees of the Order of the Sisters of Mercy in Queensland
by DAVIES COLLISION CAVE
Patent Attorneys for the Applicant

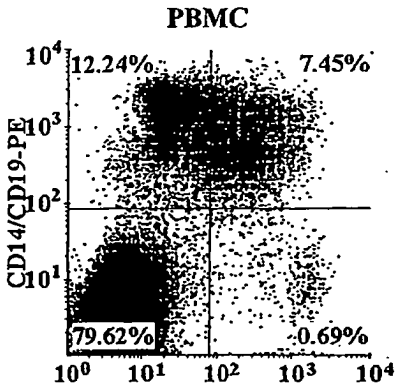

Figure 1A

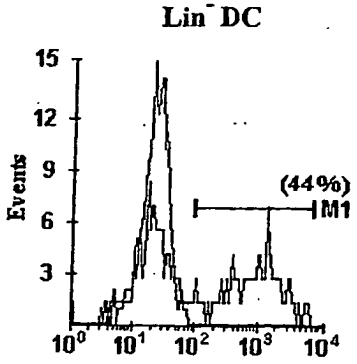

Figure 1B

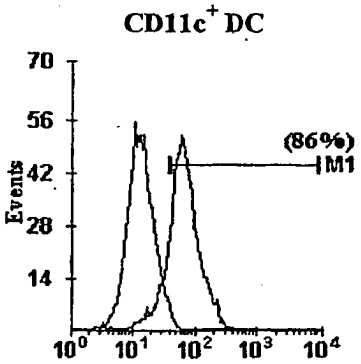

Figure 1C

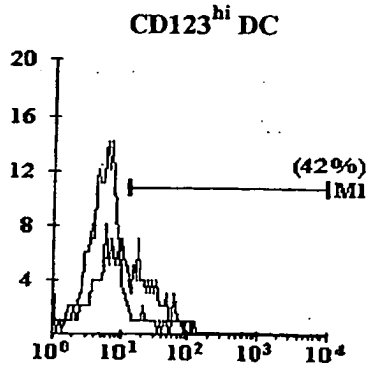

Figure 1D

[redacted]

AS only

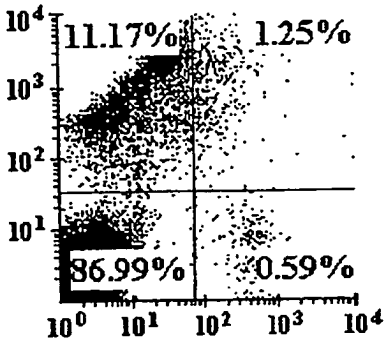

Figure 2A

CMRF-44 only

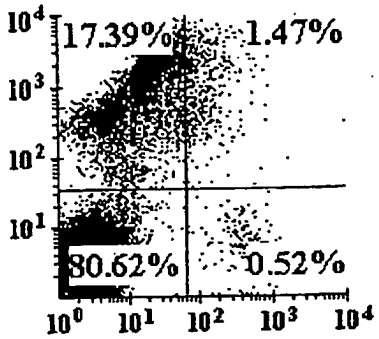

Figure 2B

CMRF-44 + AS

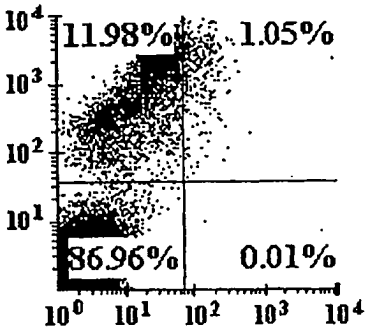

Figure 2C

IgM + AS

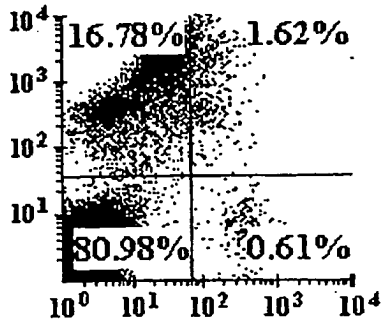

Figure 2D

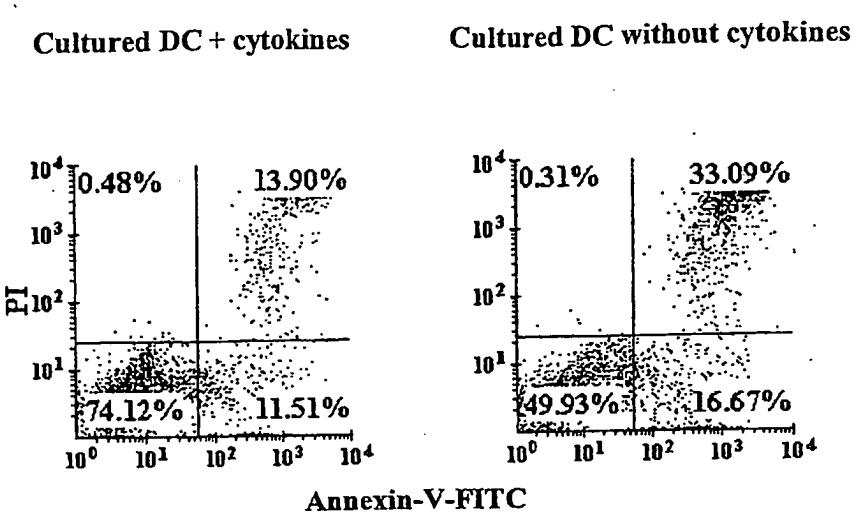

Figure 3A

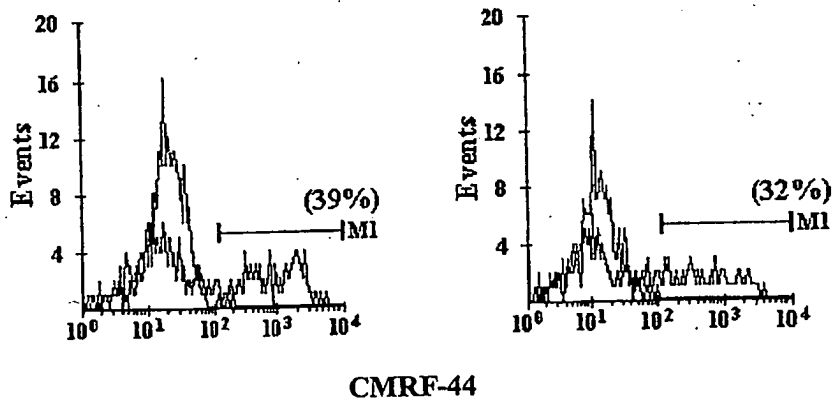

Figure 3B

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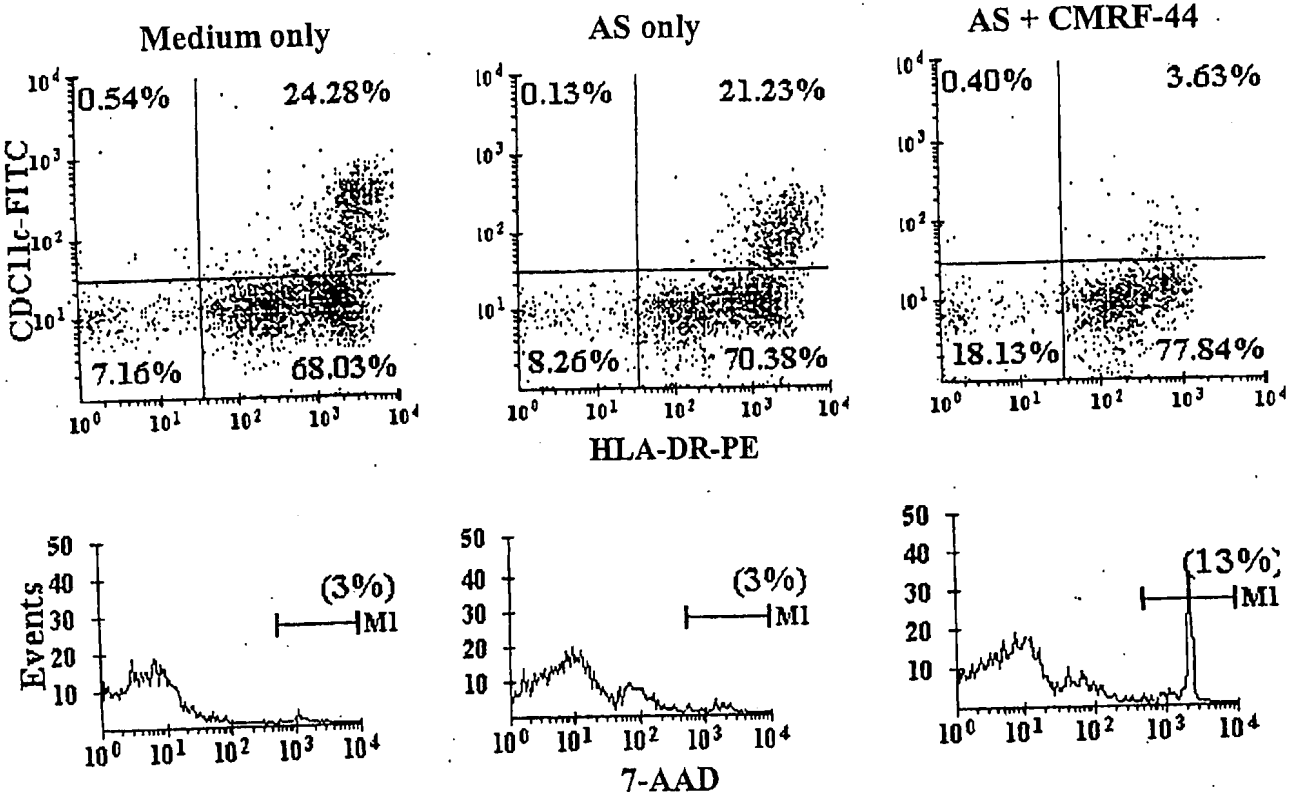


Figure 4A **Figure 4B** **Figure 4C**

CD11c^{+} cells

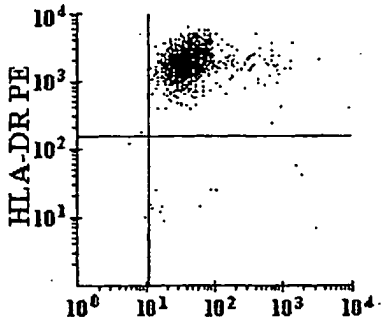


Figure 5A

CD123^{hi} cells

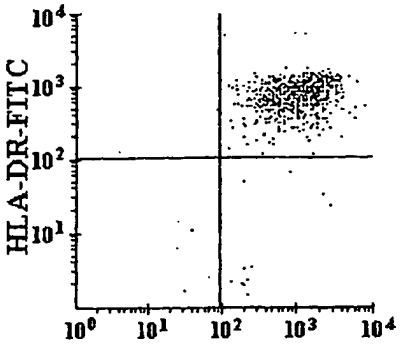


Figure 5C

CD11c^{+} cells treated with CMRF-44 + AS

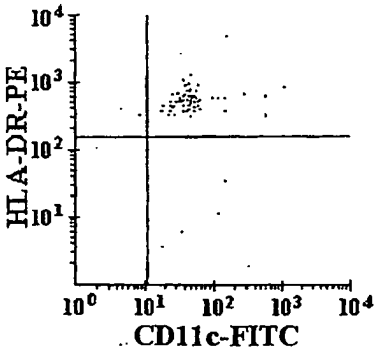


Figure 5B

CD123^{hi} cells treated with CMRF-44 + AS

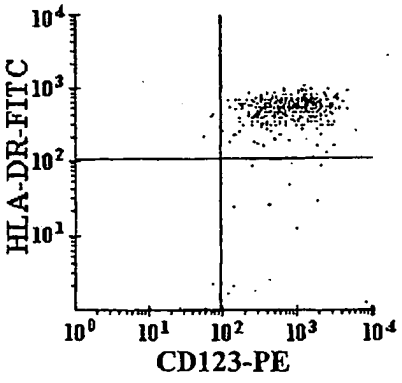


Figure 5D

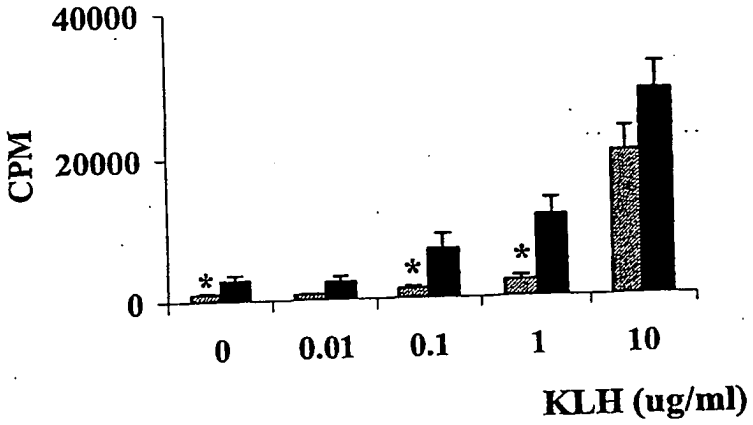


Figure 6A

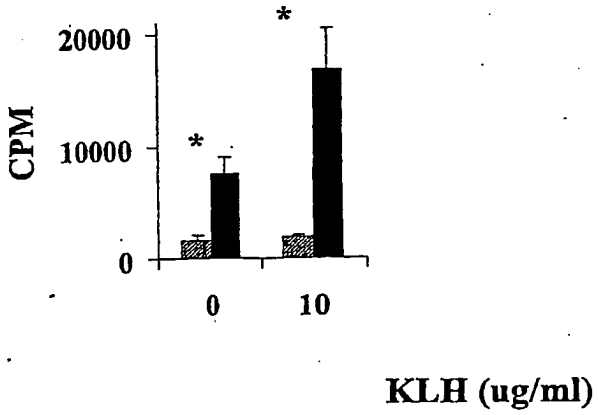


Figure 6B

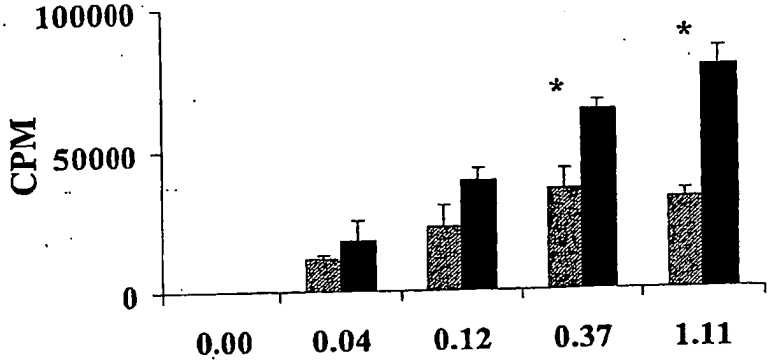
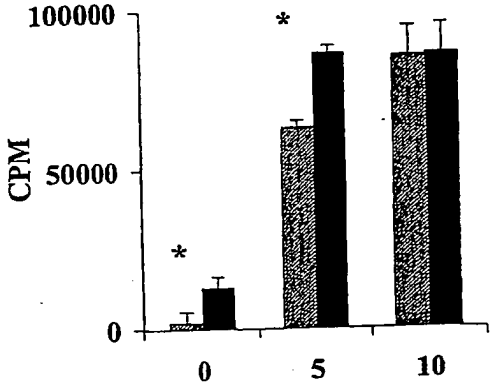
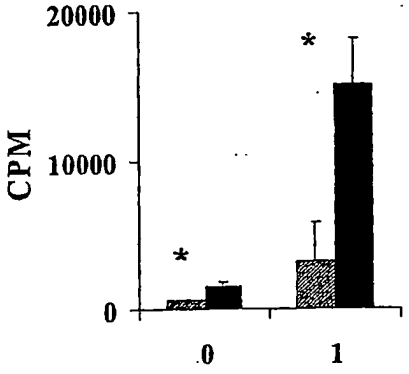


Figure 7A

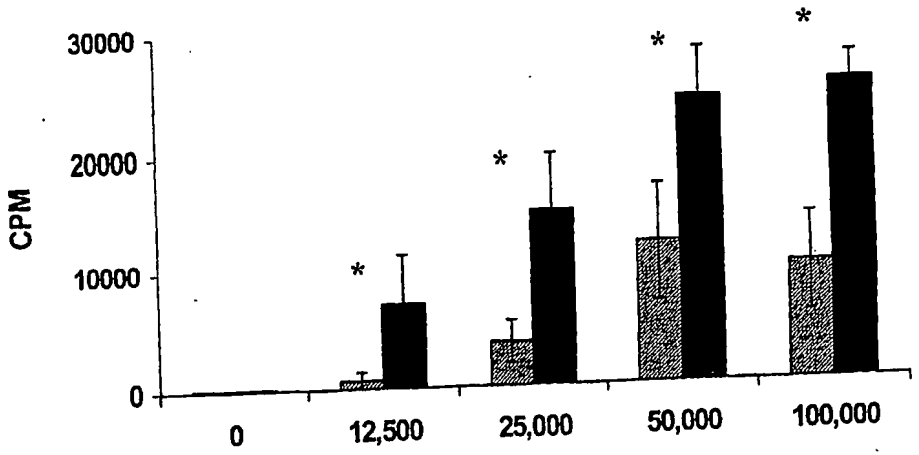




Tetanus toxoid (ug/ml)

Figure 7B

Figure 7C


30000
20000
10000
0
CPM
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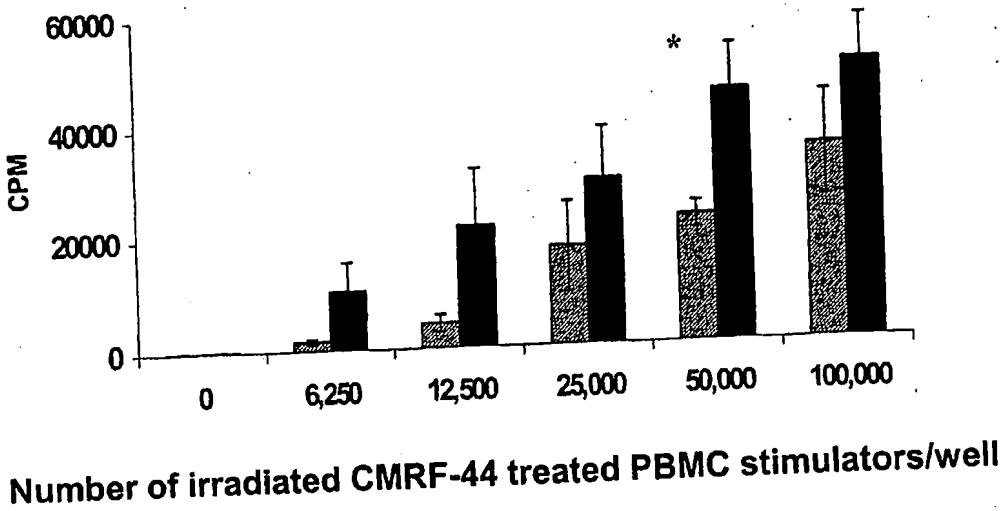
0
12,500
25,000
50,000
100,000
Number of irradiated CMRF-44 treated PBMC stimulators/well

Figure 8A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40000
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CPM
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6,250
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50,000
100,000
Number of irradiated CMRF-44 treated PBMC stimulators/well

Figure 8B